

Substituted Borosilicate Glasses with Improved Osteogenic Capacity for Bone Tissue Engineering

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Abstract

Borosilicate bioactive glasses (BBGs) have shown the capacity to promote a higher formation of new bone when compared to silicate bioactive glasses. Herein, we assessed the capacity of BBGs to induce the osteogenic differentiation of bone marrow mesenchymal stem cells (BM-MSCs), as a function of their substituted divalent cations (Mg^{2+} , Ca^{2+} , Sr^{2+}). To this purpose, we synthesized BBG particles by melt quenching. The cell viability, proliferation and morphology, (i.e. PrestoBlue[®], PicoGreen[®], and DAPI and Phalloidin stainings, respectively) as well as protein expression (i.e. alkaline phosphatase, ALP, osteopontin, OP, and osteocalcin, OC) of BM-MSCs in contact with the BBGs were evaluated for 21 days. We observed an enhanced expression of the bone-specific proteins (ALP, OP and OC) and high mineralization of BM-MSCs under BBG-Mg and BBG-Sr conditioned osteogenic media for concentrations of 20 and 50 mg/ml with low cytotoxic effects. Moreover, BBG-Sr, at a concentration of 50 mg/ml, was able to increase the mineralization and expression of the same bone-specific proteins even under basal media conditions. These results indicated that the proposed BBGs improved the osteogenic differentiation of BM-MSCs. Therefore, showing their potential as relevant biomaterials for bone tissue regeneration, not only by bonding to bone tissue, but also by stimulating new bone formation.

Key words: Borosilicate glasses, strontium, mineralization, osteogenic induction, BM-MSCs

1. Introduction

The properties of bioactive glasses (BGs) support their key relevance in clinical applications associated to bone tissue repair and regeneration. (1, 2) They are part of a tissue engineering-based strategy that can overcome the drawbacks of the traditionally used autologous bone grafts (e.g. lack of adequate amount and quality of bone, donor site morbidity). BGs are considered relevant for bone tissue repair since they: (i) promote osteointegration (forming a bone-like hydroxyapatite (HA) layer on their surface); (ii) are biocompatible; and (iii) their degradation shows positive biological effects after implantation. (2-5) There are, however, drawbacks associated with conventional BGs including *in vitro* cytotoxicity related to the release of Na⁺ ions, and interest in modified compositions has increased in recent years. (6)

Recently, borosilicate bioactive glasses (BBGs) have attracted interest in bone tissue engineering. (1, 7, 8) BBGs have shown capability to improve the new bone formation when compared to silicate-based BGs. (9, 10) They present controllable degradation rates and have a high compositional flexibility that potentially allows BBGs to be tailored with enhanced osteogenic and angiogenic properties, as well as with antibacterial capacity. (11, 12) On one hand, as shown by Huang *et al.*, a glass network composed of borosilicate's have more controllable conversion rates to HA. (13) This has also been demonstrated to occur *in vivo*. (14) As a matter of fact, the addition of borate to the glass network can also be beneficial for bone healing, as well as formation, and maintenance of new bone, while supporting cell osteogenic differentiation. (15) Frequently, it has been associated with the increase in bone resistance to fracture. (16, 17) On the other hand, by exploiting the compositional flexibility of BBGs, inorganic divalent cations, such as Mg²⁺, Ca²⁺ and Sr²⁺ can be incorporated and play a key role in bone metabolism. For instance, Mg²⁺ increases bone formation rate, as well as

stimulates bone cell adhesion increasing their stability. (18, 19) The Ca^{2+} is known to be essential during the apatite formation process, being also favorable to osteoblast proliferation, differentiation and the mineralization of the extracellular matrix (ECM). (20) Sr^{2+} also has bone therapeutic potential. Different studies evidenced its beneficial effects on bone cells and bone formation *in vivo*, (21, 22) being even used for the treatment of osteoporosis. (23)

In general BGs are known to be osteoinductive materials, capable of stimulating the function and osteogenic differentiation of bone and stem cells without any additional supplementation. Findings from Fu *et al.* suggested that the borosilicate 13-93B1 scaffolds supported the proliferation and function of osteogenic Murine Osteocyte-like Cell Line MLO-A5,(15) while Gentleman *et al.* demonstrated that Sr^{2+} -substituted BGs stimulated osteoblast metabolic activity promoting cell proliferation and ALP activity. (24) More significantly, Santocildes *et al.* demonstrated that Sr-containing BGs appeared to be capable of promoting osteoblastic differentiation in a proportion of bone marrow mesenchymal stem cells (BM-MSCs) that were in some way pre-committed to this lineage. (25) Liang *et al.* showed that borate glasses support the attachment and differentiation of human bone marrow derived mesenchymal stem cells and human mesenchymal stem cell derived osteoblasts. (26)

The present study aims to fabricate three substituted BBGs to be used for bone tissue regeneration. We evaluated the impact of incorporating different divalent cations (i.e. Mg^{2+} , Ca^{2+} , Sr^{2+}) into the BBGs on their ability to induce the BM-MSCs (known to differentiate into the mesodermal lineage cells, such as osteoblasts, osteoclasts and osteocytes (27)) to proliferate, differentiate and mineralize the ECM, while promoting bone formation. (11)

2. Experimental

2.1. Preparation of BBGs

The BBGs of general formula $0.05\text{Na}_2\text{O} \cdot x\text{MgO} \cdot y\text{CaO} \cdot (0.35-x-y)\text{SrO} \cdot 0.20\text{B}_2\text{O}_3 \cdot 0.40\text{SiO}_2$ (molar ratio, where $x, y = 0.35$ or 0.00 , and $x \neq y$) were synthesized by melt-quenching. The suitable amounts of, silica (SiO_2 , Macherey-Nagel, Germany), boron oxide (B_2O_3 , Alfa Aesar, Germany), sodium bicarbonate (NaHCO_3 , Sigma-Aldrich, Australia), and magnesium oxide (MgO , Sigma-Aldrich, Portugal), or calcium carbonate (CaCO_3 , Sigma-Aldrich, Portugal), or strontium carbonate (SrCO_3 , Sigma-Aldrich, Portugal) were thoroughly mixed with the addition of ethanol in a porcelain pestle with the help of a mortar, vacuum dried overnight and transferred to a platinum crucible. After entirely dried, each batch was heated to 1450°C in air for 1 h and subsequently the melt was quickly poured into cold water to form the glass frit. Afterwards, the as-quenched glasses were ground in an Agate mortar (RETSCH, Germany) and sieved to a particle size $<63\ \mu\text{m}$. Before the *in vitro* tests BBG-Mg ($0.05\text{Na}_2\text{O} \cdot 0.35\text{MgO} \cdot 0.20\text{B}_2\text{O}_3 \cdot 0.40\text{SiO}_2$), BBG-Ca ($0.05\text{Na}_2\text{O} \cdot 0.35\text{CaO} \cdot 0.20\text{B}_2\text{O}_3 \cdot 0.40\text{SiO}_2$) or BBG-Sr ($0.05\text{Na}_2\text{O} \cdot 0.35\text{SrO} \cdot 0.20\text{B}_2\text{O}_3 \cdot 0.40\text{SiO}_2$) were weighted, dried and sterilized at 160°C for at least 2 h.

2.2. Morphology and chemical composition of BBGs

The morphology of the synthesized BBGs was observed by scanning electron microscopy (SEM, model S360, Leica Cambridge, UK) equipped with energy dispersive X-ray spectroscopy (SEM/EDS link-eXL-II) for the determination of the surface chemical composition.

2.3. Isolation and expansion of mesenchymal stem cells

BM-MSCs were isolated from bone marrow of 4-5 week-old male Wistar rats according to the method established by Maniatopoulos *et al.* (28) and recently proposed by Santocildes *et al.* (25) BM-MSCs were expanded in basal medium consisting of Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, UK), supplemented with 100 U/ml penicillin (Sigma-Aldrich, UK) and 1 mg/ml streptomycin (Sigma-Aldrich, UK). Cells were cultured at 37 °C in an atmosphere of 5% CO₂.

Prior to the *in vitro* studies, BM-MSCs, at passage 2, were harvested and seeded into 24 well plates, at a density of 2×10^4 cells per well. Cells were cultured in the presence of the BBGs at concentrations of 20 and 50 mg/ml, for 7, 14 and 21 days under static conditions. The BM-MSCs cultured in the absence of BBGs were used as negative control and in the presence of 45S5 bioglass[®] as positive control. The BBGs at the desired concentrations and 45S5 bioglass[®] were deposited on top of the cells, in cell culture inserts with porous membranes (0.4 µm ThinCerts[™] Cell Culture Inserts; Greiner, Germany), as used in previous works. (29) The ThinCerts[™] were used as an inert platform to support the glass particles under cell culture conditions. All BBG compositions and controls were cultured in basal and osteogenic differentiation media (basal medium supplemented with 50 µg/ml ascorbic acid, 10 mM β-glycerophosphate and 10⁻⁸ M dexamethasone). Figure 1 presents a schematic of the experimental design.

Figure 1. Schematic of the experimental design.

2.4. Potential cytotoxic effect of BBGs dissolution on BM-MSCs

Cell viability and proliferation (PrestoBlue[®] and PicoGreen[®] assays). The PrestoBlue[®] reagent (Fisher Scientific, UK) is a resazurin-based solution that is reduced to resorufin by viable cells which can be detected fluorimetrically. The cell viability

assay was executed according to the manufacturer's instructions. In brief, the PrestoBlue[®] reagent was added to a final concentration of 10% to the wells and the cells were incubated for 1 h at 37 °C. Afterwards, 200 µl samples of the culture medium were removed and placed in 96-well plates and the resorufin fluorescence was quantified spectrophotometrically using a plate reader (Tecan Infinite M200). The fluorescence was determined at an excitation wavelength of 560 nm and emission wavelength of 590 nm. The metabolic activity was presented in fluorescence values and compared with the control (cell cultured in the absence of glass particles under basal medium conditions).

The PicoGreen[®] dsDNA reagent (Invitrogen, USA) is an ultrasensitive fluorescent nucleic acid dye for quantification of double-stranded DNA (dsDNA) in solution. This assay enables the measurement of cell proliferation. After each culturing period, the cell monolayers were washed with PBS and then incubated at 37 °C for 3 h followed by a freezing step at -80 °C for at least overnight in ultra-pure water (1 ml) to ensure cell lysis. The assay was performed according to the manufacturer's protocol. And the fluorescence was determined at an excitation wavelength of 485 nm and emission wavelength of 528 nm. The DNA concentration was presented in µg/ml and compared with the control (cell cultured in the absence of glass particles under basal medium conditions).

Cell morphology and distribution. After each culturing period the cell grown in tissue culture coverslips were washed with PBS and fixed with 4% formalin solution (0.5 ml) for 15 min at room temperature (RT). The cell layers were then washed with PBS, containing 0.2% Triton X, for 2 min. After the fixation and permeation steps, the cell monolayers were washed again with PBS and stained with 4,6-diamidino-2-phenylindole dilactate (1:1000 DAPI, Sigma, UK) for 2 min at RT, and phalloidin-tetramethylrhodamine B isothiocyanate (Sigma, UK) for 1 h at RT. Finally, the cells

were washed and observed using an Axioplan 2 imaging fluorescent microscope with a digital camera QIC AM 12-bit (Zeiss, UK).

2.5. Osteogenic capacity of BBGs on BM-MSCs

Alkaline phosphatase quantification. The concentration of alkaline phosphatase (ALP) was determined for all the culture time periods, using the lysates used for DNA quantification. Briefly, the ALP quantity was assessed using the Alkaline Phosphatase, Diethanolamine Detection kit (Sigma-Aldrich, UK) in which p-nitrophenyl phosphatase (pNPP) solution is hydrolyzed by ALP to yellow free p-nitrophenol. In brief, a buffered pNPP solution was prepared and equilibrated at 37 °C. Afterwards, 2% (v/v) of sample or control were added. Immediately after mixing the absorbance was read at 405 nm in a plate reader (Tecan Infinite M200) for ≈ 5 min. An ALP standard solution was used as control and buffer as blank. The units were calculated according to the following equation:
$$\frac{(\Delta A_{405nm} / \text{min Test} - \Delta A_{405nm} / \text{min Blank}) \times df \times V_F}{18.5 \times V_E}$$
 Where df = dilution factor; V_F = Volume of final solution; 18.5 = millimolar extinction coefficient of pNPP at 405 nm and V_E = Volume of samples/ALP standard solution. ALP activity was calculated by normalizing ALP concentration per DNA concentration for each condition and time point.

Alizarin red staining. After 21 days of culture, the cells grown in tissue culture coverslips were fixed in 70% ice-cold methanol at -20 °C at least for 30 min. The cell layers were then washed with PBS and dried overnight. Afterwards, cells were stained with alizarin red solution [342 mg of alizarin red, (Sigma-Aldrich, UK) in 25 ml of distilled water and the pH was adjusted to 4.1 with 10% ammonium hydroxide (Sigma-Aldrich, UK)] for 10 min. Afterwards, the coverslips were washed with distilled water, dehydrated in an acetone/xylene (Sigma-Aldrich, UK) mixture and mounted using an

aqueous mountant. The stained constructs were observed under an optical microscope (BX51, Olympus Corporation, UK) and images were captured by a digital camera (DP70, Olympus Corporation, UK). The BM-MSCs morphology and mineral deposition was also observed using SEM (model S360, Leica Cambridge, UK) equipped with energy dispersive X-ray spectroscopy (SEM/EDS link-eXL-II) for the determination of the surface chemical composition.

Immunodetection of bone-specific proteins. Osteopontin (OP) and osteocalcin (OC) protein expression of BM-MSCs was assessed by immunoassay technique to evaluate their osteoblastic differentiation. The procedures were executed according to the manufacturer's instructions. The concentrations of OP and OC were determined for all the culture time periods, using the lysates used for DNA quantification. The OP quantitative determination was performed using Mouse/Rat Osteopontin Quantikine ELISA Kit (R&D Systems, UK). In brief, 50 μ l of assay diluent RD1W and 50 μ l of standard (2500 to 39 pg/ml), control and samples were added and the plate incubated for 2 h at RT. After 4 washing steps and perfectly dried, 100 μ l of Mouse/Rat OP Conjugated were added and incubated for 2 h at RT. The sandwich complex was washed 4 times and allowed to react with 100 μ l of substrate solution before adding 100 μ l of stop solution. Finally, the optical density was determined at 450 nm and the concentration of OP obtained from a standard curve plot. OC quantitative determination was performed by the use of Rat Gla-Osteocalcin High Sensitive EIA kit (Takara Clontech, Japan). In brief, 100 μ l of samples and standard solution (16 to 0.25 ng/ml) were incubated for 1 h at 37 °C with the capture-antibody, rat osteocalcin C-terminus-specific antibody. After OC capture and 3 washing steps, 100 μ l of the enzyme-labelled antibody (GlaOC4-30) specific to Gla-OC was incubated for 1 h at RT. The sandwich complex was washed 4 times and allowed to react with 100 μ l of substrate solution for

10-15 min. Finally, after adding the stop solution the optical density was determined at 450 nm and the concentration of OC obtained from a standard curve plot. OP and OC content was calculated by normalizing OP or OC concentration per DNA concentration for each condition and time point.

2.6. Statistical analysis

Results are expressed as mean \pm standard deviation with $n = 3$ for each sample. Error bars represent standard deviations. The data was analyzed by non-parametric statistics: Kruskal-Wallis test ($p < 0.0001$), followed by a Dunn's Multiple Comparison test. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ in respect to the control.

3. Results and discussion

3.1 Morphology of BBGs and their chemical composition

BBGs' frits were successfully obtained by melt quenching and ground in a controlled manner. Figure 2a, 2b and 2c show the SEM/EDS analysis of BBGs, which exhibited an angular shape with low sphericity and confirmed the successful incorporation of the different modifier divalent cations (i.e. Mg^{2+} , Ca^{2+} and Sr^{2+}). The composition of BBGs was confirmed in a prior work by the use of X-ray fluorescence and follows the general formula $0.05Na_2O \cdot xMgO \cdot yCaO \cdot (0.35-x-y)SrO \cdot 0.20B_2O_3 \cdot 0.40SiO_2$ (molar ratio, where x , $y = 0.35$ or 0.00 , and $x \neq y$). (30) Moreover, studies after immersion in SBF, showed that the studied BBGs are bioactive due to the formation of bone-like apatite structures onto their surface, and the constant release of ions to the reaction media over time. (29)

Figure 2. SEM/EDS micrographs of BBGs, a) BBG-Mg, b) BBG-Ca and c) BBG-Sr. SEM images are shown as insets, displaying the morphology of the glass particles. The specific modifier divalent cation is highlighted in yellow for each BBG.

3.2 *In Vitro* biological evaluation

Osteoblast differentiation can be divided into three stages: cell proliferation, ECM synthesis and maturation, and ECM mineralization, each with a cellular characteristic behavior. (31)

3.2.1 Potential cytotoxic effect of BBGs leachables on BM-MSCs

The effect of BBGs' concentration on cell viability and proliferation was studied on a previous work. (29, 30) It showed that a concentration between 20 and 50 mg/ml did not significantly affect cell viability and proliferation. On the other hand, Romero *et al.* (32) studied the osteogenic response of BM-MSCs to strontium-substituted bioactive glasses (SrBG) and observed that 20 mg of Sr50BG promoted the osteoblastic differentiation of BM-MSCs. Based on these results, we decided to use BBGs concentrations of 20 and 50 mg/ml.

Herein, we cultured BM-MSCs in basal and osteogenic differentiation media for 7, 14 and 21 days under static conditions, in the presence and absence of BBGs, in order to evaluate their biological activity. The cellular metabolism and proliferation was evaluated by quantifying the conversion of resazurin to resorufin by viable cells and the amount of double stranded DNA (live cells) in the culture wells; as well as their morphology.

Fluorescence microscopy images showed the morphology of BM-MSCs in culture containing BBGs or 45S5 bioglass[®] (Figure 3). The adhered BM-MSCs exhibited a well-spread morphology, exhibiting cell-to-cell contacts in a comparable manner on the

BBGs conditioned cultures as in the control experiment. While BM-MSCs cultured with 45S5 bioglass[®] presented a round shape for the last time point (21 days), suggesting cell death. At the same timepoint, especially for cultures under osteogenic differentiation media (e.g. BBG-Sr), there was distinguishable well-spread polygonal shape cells, suggesting osteoblast-like morphology.

Figure 3. BM-MSCs morphology observed by fluorescence microscopy, after 7, 14 and 21 days culture with BBGs either under basal or osteogenic culture medium. Each sample was incubated at two different concentrations (20 and 50 mg/ml). Cells cultured with basal and osteo medium were used as negative control and 45S5 bioglass[®] incubated with medium was used as positive control. Nuclei stained blue by DAPI; Actin stained green by Phalloidin.

Figure 4. Metabolic activity (PrestoBlue[®] assay) and proliferation (PicoGreen[®] assay) of BM-MSCs cultured either in basal or osteogenic media in the presence of different concentrations (20 and 50 mg/ml) of BBG-Mg (a, b), BBG-Ca (c, d) and BBG-Sr (e, f). The 45S5 bioglass[®] (g, h) was used as control. Standard culture medium was used as negative control. Results are expressed as mean \pm standard deviation with $n = 3$ for each bar. The data was analyzed by non-parametric statistics: Kruskal-Wallis test ($p < 0.0001$), followed by a Dunn's Multiple Comparison test. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ in respect to the control, i.e. BM-MSCs culture in basal media for the respective day of culture (i.e. 7, 14 and 21 days).

From the PrestoBlue[®] data (Figure 4a, 4c, 4e and 4g) it is possible to observe that the metabolic activity of BM-MSCs increased over the 21 days of culture. In the first 7 days of culture, the BM-MSCs under conditioned cultures presented reduced viability in respect to the cells culture under basal condition (negative control), especially for BBG-Ca culture conditions, which may be due to the initial burst release of ions to the media. In contrast, from day 14, BM-MSC cultures under osteogenic media displayed a reduction of metabolic activity with respect to the control (absence of glass particles and cell cultured under basal medium). It is noteworthy that BM-MSC cultures at day 21 with 45S5 bioglass[®] under osteogenic media showed very low metabolic activity when compared with the control experiment (osteogenic media), being consistent with the cell death observed by fluorescent microscopy (Figure 3). Complementary to the viability analysis, the cellular proliferation was also assessed by measuring the total cell DNA (Figure 4b, 4d, 4f, 4h). The PicoGreen[®] data showed an increase on the number of BM-MSCs over time. However, under osteogenic media, the cells presented lower proliferation rates than cultures under basal media from day 14. Noteworthy, is the prominent reduction of BM-MSCs for cultures with BBG-Mg (osteogenic and basal media) and 45S5 bioglass[®] (osteogenic media) at day 21. To emphasize, BBG-Sr and 45S5 bioglass[®] under osteogenic media showed a significant reduction on the cell proliferation at day 14 when compared with the cells cultured under basal media, suggesting an alteration of biological behavior. (33) Regarding the effect of concentration, there was no potential toxic effects with time except in the case of BM-MSCs cultured in the presence of BBG-Mg (osteogenic and basal media) and 45S5 bioglass[®] (osteogenic media) at day 21, where a large reduction in cell number was observed in relation to the control (basal media).

3.2.2 Alkaline phosphate quantification

It is commonly accepted that ALP is a key player in the process of osteogenesis, being ALP known to be involved in early stages of normal and pathological calcification. (34-36) In general, an increase of the ALP activity is correlated with osteogenesis, increasing during the bone formation stage. (37) Not surprisingly, the ALP quantification data (Figure 5) showed a significantly higher ALP activity when cells were cultured under osteogenic media rather than basal media. (38) However, at day 21 the levels of ALP activity on BBG-Mg, -Sr and 45S5 bioglass[®] cultured under osteogenic media are significantly higher than the cultures without glass addition (e.g. at day 21, under osteogenic media, the addition of 20 mg of BBG-Sr (c) induced a highly significant ($p<0.001$) enhanced ALP activity in relation to the cells cultured in the absence of glass particles). BBG-Mg, -Sr and 45S5 bioglass[®] were not capable of inducing the ALP protein expression alone, however, they were capable to increase the ALP expression during the differentiation process of BM-MSCs into osteoblasts under osteogenic media (for 21 days of cell culture). It is relevant to point out that previous studies quantified the chemical species released from BBGs in solution, demonstrating a higher concentration of Mg^{2+} and Sr^{2+} ions (2-fold) when compared with Ca^{2+} (from BBG-Mg, BBGs-Sr and BBG-Ca, respectively). (29, 30) The presence of higher concentration of specific ions (i.e. Mg^{2+} and Sr^{2+}) might facilitate cell differentiation. It is also relevant to highlight the fact that along with the increase of ALP activity for the case of BBG-Mg and -Sr glasses there was observed an increase of cell proliferation. In contrast, the viability and proliferation data, presented a reduction of viable and live cells for the BM-MSCs cultured in the presence of these BBG-Ca and 45S5 bioglass[®]. Remarkably, BBG-Sr promoted the increase of ALP activity for both concentrations of glass particles, i.e. 20 and 50 mg/ml.

Figure 5. ALP activity of BM-MSCs (cultured either in basal or osteogenic media) in the presence of different concentrations (0, 20 and 50 mg/ml) of BBG-Mg (a), BBG-Ca (b) and BBG-Sr (c). The 45S5 bioglass[®] (d) was used as control. Results are expressed as mean \pm standard deviation with $n = 3$ for each bar. The data was analyzed by non-parametric statistics: Kruskal-Wallis test ($p < 0.0001$), followed by a Dunn's Multiple Comparison test. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ in respect to the control, i.e. BM-MSCs culture in basal media for the respective day of culture (i.e. 7, 14 and 21 days).

3.2.3 Matrix mineralization

Similar results were found at day 21 after alizarin red staining for mineral deposits (Figure 6). In agreement with ALP activity data, cells cultured under osteogenic media yielded more bone-like nodules (intense red spots dispersed in the cell culture), resulting from ECM mineralization. (35) Of importance, and corroborating with ALP activity data, mineral deposits were more evident with BBG-Mg, -Sr and 45S5 bioglass[®] (Figure 6a, 6c and 6d) and an increase of red nodules with the increase of concentrations can be observed in the case of the cells cultured in the presence of BBG-Sr (Figure 6c, osteo 20 mg/ml and 50 mg/ml). Higher concentrations of BBG-Sr and 45S5 bioglass[®] show the presence of red nodules even in cells cultured in basal medium. However, ALP activity data, suggests that BBG-Sr and 45S5 bioglass[®] (Figure 6c and 6d basal for a concentration of 50 mg/ml) are capable to induce ECM mineralization by themselves. Therefore, the combination of ALP activity and mineralization results suggest the use of BBG-Sr to promote osteogenesis. (39)

Figure 6. Alizarin red staining of BM-MSCs cultured during 21 days, either in basal or osteogenic media in the presence of different concentrations (20 and 50 mg/ml) of BBG-Mg (a), BBG-Ca (b) and BBG-Sr (c). The 45S5 bioglass[®] (d) was used as control.

Figure 7. SEM micrographs of BM-MSCs in the presence of different concentrations (20 and 50 mg/ml) of BBG-Mg (a), BBG-Ca (b) and BBG-Sr (c) after 21 days of cell culture either in basal or osteogenic media. Higher SEM magnifications are shown as insets, highlighting the formed apatite-like structures. The 45S5 bioglass[®] was used as a control.

In Figure 7 we presented SEM micrographs of BM-MSCs cultured for 21 days in the presence of BBGs and 45S5 bioglass[®]. In the images it is possible to observe the deposition of minerals over the dense layer of cells, when they were cultured in the presence of BBG-Mg, -Sr and 45S5 bioglass[®] (Figure 7a, 7c and 7d). The presence of these glass compositions in the culture medium promoted a mineralization typical to occur while BM-MSCs differentiate into osteoblasts. (38) This mineralization is in agreement with the alizarin red data where the mineral deposits were more evident in the cells cultured in the presence of BBG-Mg, -Sr and 45S5 bioglass[®] (Figure 6a, 6c and 6d). In these culture conditions, it is observed in the SEM/EDS images calcium phosphate deposits over the dense cellular layer. Once more, higher concentrations of BBG-Sr and 45S5 bioglass[®] under basal culture conditions promoted the deposition of a higher amounts of calcium phosphate structures, suggesting that BBG-Sr and 45S5 bioglass[®] (Figure 7c and 7d basal for a concentration of 50 mg/ml) are capable of inducing ECM mineralization by themselves, which could be beneficial for bone regeneration. (39)

3.2.4 Protein expression (OP and OC)

Complementary to the reported biological data, the differentiation level of BM-MSC, cultured in the presence (20 and 50 mg/ml) and absence of BBG-Mg, -Ca, -Sr and 45S5 bioglass[®] (either in basal or osteogenic media) was assessed by the quantification of the expression level of two major bone-specific proteins, i.e. OP and OC. The relative expression of these proteins was normalized in relation to the number of cells, i.e. amount of dsDNA. It is well known that osteoblasts are differentiated cells that mineralize the bone matrix. OP is a phosphoprotein synthesized by bone forming cells, which present calcium-binding domains and is responsible for cell attachment, proliferation, and ECM mineralization. (40) In the case of OC, it is a bone-specific glycoprotein capable of binding to calcium, which promotes ECM calcification. (40) Not surprisingly, the OP and OC quantification data (Figure 8 and Figure 9) showed a significantly higher protein expression when BM-MSCs were cultured under osteogenic media rather than basal media. (41) In the case of OP, as expected, a delay in the protein synthesis is observed (Figure 8). At day 7 there was no significant difference of OP expression in relation to the control (absence of glass particles and cell culture in basal medium). However, at day 14 there is a high expression peak by BM-MSCs cultured in osteogenic medium (in the presence of BBG-Mg, -Sr and 45S5 bioglass[®]), which determines the decay of the matrix deposition phase and the beginning of the mineralization phase. Moreover, BBG-Sr and 45S5 bioglass[®] continue to induce a significant overexpression of OP over time (e.g. at day 21), supporting the mineralization demonstrated by ALP and alizarin red analysis (Figure 5 and 6, respectively). In the OC case there was a high protein expression up to day 14, indicating bone ECM maturation (Figure 9). (42) At day 7 there is a significant difference in OP expression in relation to the control (cell culture in basal medium and

in the absence of glass particles). After day 7 there was a reduction of OC expression, consistent with matrix mineralization. Noteworthy is the observation that BBG-Sr under basal medium induced the BM-MSCs to exhibit a peak of OC expression at day 14. This data suggested that the BBG-Sr glass particles (at a concentration of 50 mg/ml) induced the OC protein expression, which is in agreement with the ALP and alizarin red data. Also, BBG-Sr and 45S5 bioglass[®] prolonged the OC overexpression over the 21 days of culture. In addition, the 45S5 bioglass[®] promoted a high deposition of OC at day 21 (Figure 4h) compared with high BM-MSCs density in the case of BBG-Sr (Figure 4f). However, it is important consider that in the case of the cultures in the presence of 45S5 bioglass[®] a very low BM-MSCs cell density was observed, which might be related with the cytotoxicity of 45S5 bioglass[®]. (43) Therefore, and overall, our data suggests that the BBG-Sr glass particles are able to induce the BM-MSCs to express higher levels of OP and OC, while maintaining the BM-MSCs cell density.

Figure 8. OP protein content of BM-MSCs cultured either with basal or osteogenic media in the presence of different concentrations (20 and 50 mg/ml) of BBG-Mg (a), BBG-Ca (b) and BBG-Sr (c). The 45S5 bioglass[®] (d) was used as control. Results are expressed as mean \pm standard deviation with $n = 3$ for each bar. The data was analyzed by non-parametric statistics: Kruskal-Wallis test ($p < 0.0001$), followed by a Dunn's Multiple Comparison test. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ in respect to the control, i.e. BM-MSCs culture in basal media for the respective day of culture (i.e. 7, 14 and 21 days).

Figure 9. OC protein content of BM-MSCs cultured either with basal or osteogenic media in the presence of different concentrations (20 and 50 mg/ml) of BBG-Mg (a), BBG-Ca (b) and BBG-Sr (c). The 45S5 bioglass® (d) was used as control. Results are expressed as mean \pm standard deviation with $n = 3$ for each bar. The data was analyzed by non-parametric statistics: Kruskal-Wallis test ($p < 0.0001$), followed by a Dunn's Multiple Comparison test. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ in respect to the control, i.e. BM-MSCs culture in basal media for the respective day of culture (i.e. 7, 14 and 21 days).

Hence, combining the obtained biological data, i.e. viability, proliferation, mineralization and protein expression analysis (ALP, OC and OP), we were able to identify the BBG-Mg, -Sr (at concentrations of 20 to 50 mg/ml) as relevant promoters of the osteogenic differentiation of BM-MSCs. Several authors reported that Mg^{2+} ions significantly enhanced osteoblast adhesion by the altering cell-matrix interactions, which modulate the function of integrins related with cell differentiation. (19, 44) Our results suggest that BBG-Mg also promoted BM-MSCs differentiation, which might be related with the presence of Mg^{2+} in the culture medium. Remarkably, BBG-Sr (at a concentration of 50 mg/ml and 21 days of culture) presented the capacity to induce osteogenic response in BM-MSCs in the absence of osteogenic medium. Other authors reported Sr containing glasses to stimulate osteoblast metabolic activity, inhibiting osteoclast differentiation, as well as promoting the increment of ALP activity. (24) For instance, Hurtel-Lemaire *et al.* (45) have shown that Sr induces osteoclast apoptosis at concentrations higher than 9 mM. This is in accordance with our previous studies (29) that demonstrated a concentration of Sr in the culture media of about 20 mM after 3

days of culture. The ALP activity results showed the shift of BM-MSCs to a more differentiated state, while the alizarin red analysis demonstrated that the cells in the presence of BBG-Mg and BBG-Sr glass particles present intense and dispersed red spots in the cell culture, corresponding to the mineralization promoted by the BM-MSCs. Finally, Santocildes *et al.* (25) demonstrated that the dissolution of Sr-containing glasses stimulated the upregulation of genes associated with the process of osteogenic differentiation, such as *Bglap* (OC) and *Spp1* (OP). In agreement with this data, we also observed that BBGs-Mg and BBG-Sr might also influence the ECM maturation and mineralization, through the promotion of the OP and OC protein overexpression, which suggests that these glass compositions may be effective in inducing and sustaining the osteoblastic phenotype. (46)

4. Conclusion

BBGs with different substituted divalent cations (Ca^{2+} , Sr^{2+} or Mg^{2+}) were successfully synthesized by melt quenching. *In vitro* studies demonstrated that the studied BBGs exhibit the capability to improve the osteogenic differentiation of BM-MSCs with no deleterious effects over cell viability and proliferation. Specially, BBG-Mg and BBG-Sr (at 20 and 50 mg/ml) provided favorable conditions for BM-MSCs to differentiate to osteoblast-like cells and induce the formation of a high amount of mineralized nodules. The phenotypic expression of two major bone-specific proteins, namely, OP and OC confirmed the osteogenic potential of the BBGs.

The findings that the BBGs are able to promote *in vitro* cell differentiation into an osteogenic lineage, support their potential application in regenerative medicine. Based on these promising results we propose the incorporation of these BBGs into biomaterials for bone regeneration. The proposed BBGs are also relevant candidates for further *in vivo* evaluation.

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Author Disclosure Statement

No competing financial interests exist.

References

1. Rahaman, M.N. 3 - Bioactive ceramics and glasses for tissue engineering. In: Boccaccini A.R., Ma P.X., eds. *Tissue Engineering Using Ceramics and Polymers* (Second Edition): Woodhead Publishing; 2014. pp. 67.
2. Rabiee, S.M., Nazparvar, N., Azizian, M., Vashae, D., and Tayebi, L. Effect of ion substitution on properties of bioactive glasses: A review. *Ceram Int* **41**, 7241, 2015.
3. Hench, L. The story of Bioglass. *Journal of Materials Science: Materials in Medicine* **17**, 967, 2006.

4. Bhakta, S., Faira, P., Salata, L., de Oliveira Neto, P., Miller, C., van Noort, R., Reaney, I., Brook, I., and Hatton, P. Determination of relative in vivo osteoconductivity of modified potassium fluorrichterite glass–ceramics compared with 45S5 bioglass. *Journal of Materials Science: Materials in Medicine* **23**, 2521, 2012.
5. Gomes, F.O., Pires, R.A., and Reis, R.L. Aluminum-free glass-ionomer bone cements with enhanced bioactivity and biodegradability. *Materials Science and Engineering: C* **33**, 1361, 2013.
6. Wallace, K.E., Hill, R.G., Pembroke, J.T., Brown, C.J., and Hatton, P.V. Influence of sodium oxide content on bioactive glass properties. *J Mater Sci: Mater Med* **10**, 697.
7. Pires, R.A., Abrahams, I., Nunes, T.G., and Hawkes, G.E. Multinuclear magnetic resonance studies of borosilicate glasses for use in glass ionomer cements: incorporation of CaO and Al₂O₃. *Journal of Materials Chemistry* **16**, 2364, 2006.
8. Pires, R.A., Abrahams, I., Nunes, T.G., and Hawkes, G.E. The role of alumina in aluminoborosilicate glasses for use in glass-ionomer cements. *Journal of Materials Chemistry* **19**, 3652, 2009.
9. Pan, H.B., Zhao, X.L., Zhang, X., Zhang, K.B., Li, L.C., Li, Z.Y., Lam, W.M., Lu, W.W., Wang, D.P., Huang, W.H., Lin, K.L., and Chang, J. Strontium borate glass: potential biomaterial for bone regeneration. *J R Soc Interface* **7**, 1025, 2010.
10. Rahaman, M.N., Day, D.E., Sonny Bal, B., Fu, Q., Jung, S.B., Bonewald, L.F., and Tomsia, A.P. Bioactive glass in tissue engineering. *Acta Biomaterialia* **7**, 2355, 2011.
11. Hoppe, A., Gldal, N.S., and Boccaccini, A.R. A review of the biological response to ionic dissolution products from bioactive glasses and glass-ceramics. *Biomaterials* **32**, 2757, 2011.
12. Lakhkar, N.J., Lee, I.-H., Kim, H.-W., Salih, V., Wall, I.B., and Knowles, J.C. Bone formation controlled by biologically relevant inorganic ions: Role and controlled

delivery from phosphate-based glasses. *Advanced Drug Delivery Reviews* **65**, 405, 2013.

13. Huang, W., Day, D., Kittiratanapiboon, K., and Rahaman, M. Kinetics and mechanisms of the conversion of silicate (45S5), borate, and borosilicate glasses to hydroxyapatite in dilute phosphate solutions. *Journal of Materials Science: Materials in Medicine* **17**, 583, 2006.

14. Yao, A., Deping Wang, and Huang, W. In Vitro Bioactive Characteristics of Borate-Based Glasses with Controllable Degradation Behavior. *J Am Ceram Soc* **1**, 303, 2006.

15. Fu, Q., Rahaman, M.N., Bal, B.S., Bonewald, L.F., Kuroki, K., and Brown, R.F. Silicate, borosilicate, and borate bioactive glass scaffolds with controllable degradation rate for bone tissue engineering applications. II. In vitro and in vivo biological evaluation. *JOURNAL OF BIOMEDICAL MATERIALS RESEARCH A* | OCT 2010 **95A**2010.

16. Shen, Y., Liu, W., Wen, C., Pan, H., Wang, T., Darvell, B.W., Lu, W.W., and Huang, W. Bone regeneration: importance of local pH-strontium-doped borosilicate scaffold. *J Mater Chem* **22**, 8662, 2012.

17. Chapin, R., Ku, W., Kenney, M., and McCoy, H. The effects of dietary boric acid on bone strength in rats. *Biol Trace Elem Res* **66**, 395, 1998.

18. Yamasaki, Y., Yoshida, Y., Okazaki, M., Shimazu, A., Uchida, T., Kubo, T., Akagawa, Y., Hamada, Y., Takahashi, J., and Matsuura, N. Synthesis of functionally graded MgCO₃ apatite accelerating osteoblast adhesion. *Journal of Biomedical Materials Research* **62**, 99, 2002.

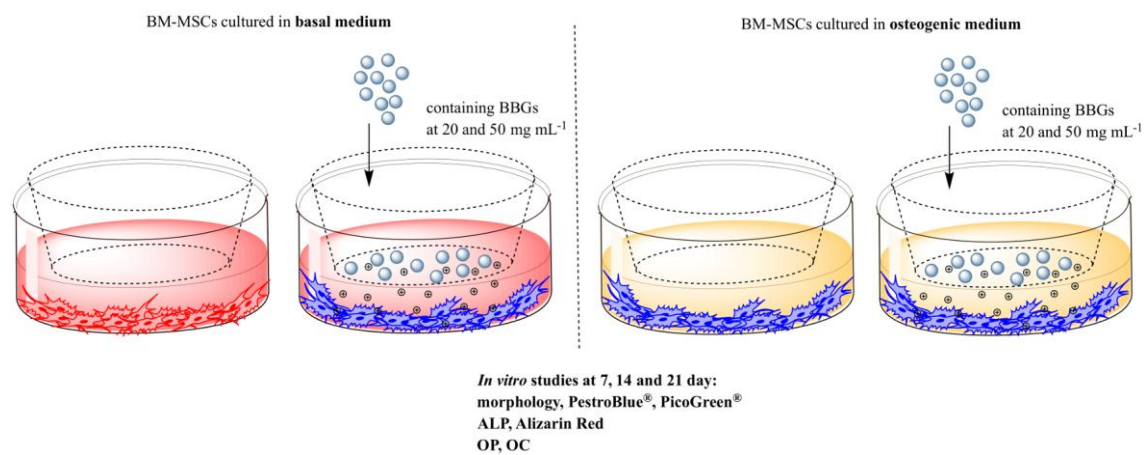
19. Zreiqat, H., Howlett, C.R., Zannettino, A., Evans, P., Schulze-Tanzil, G., Knabe, C., and Shakibaei, M. Mechanisms of magnesium-stimulated adhesion of osteoblastic cells

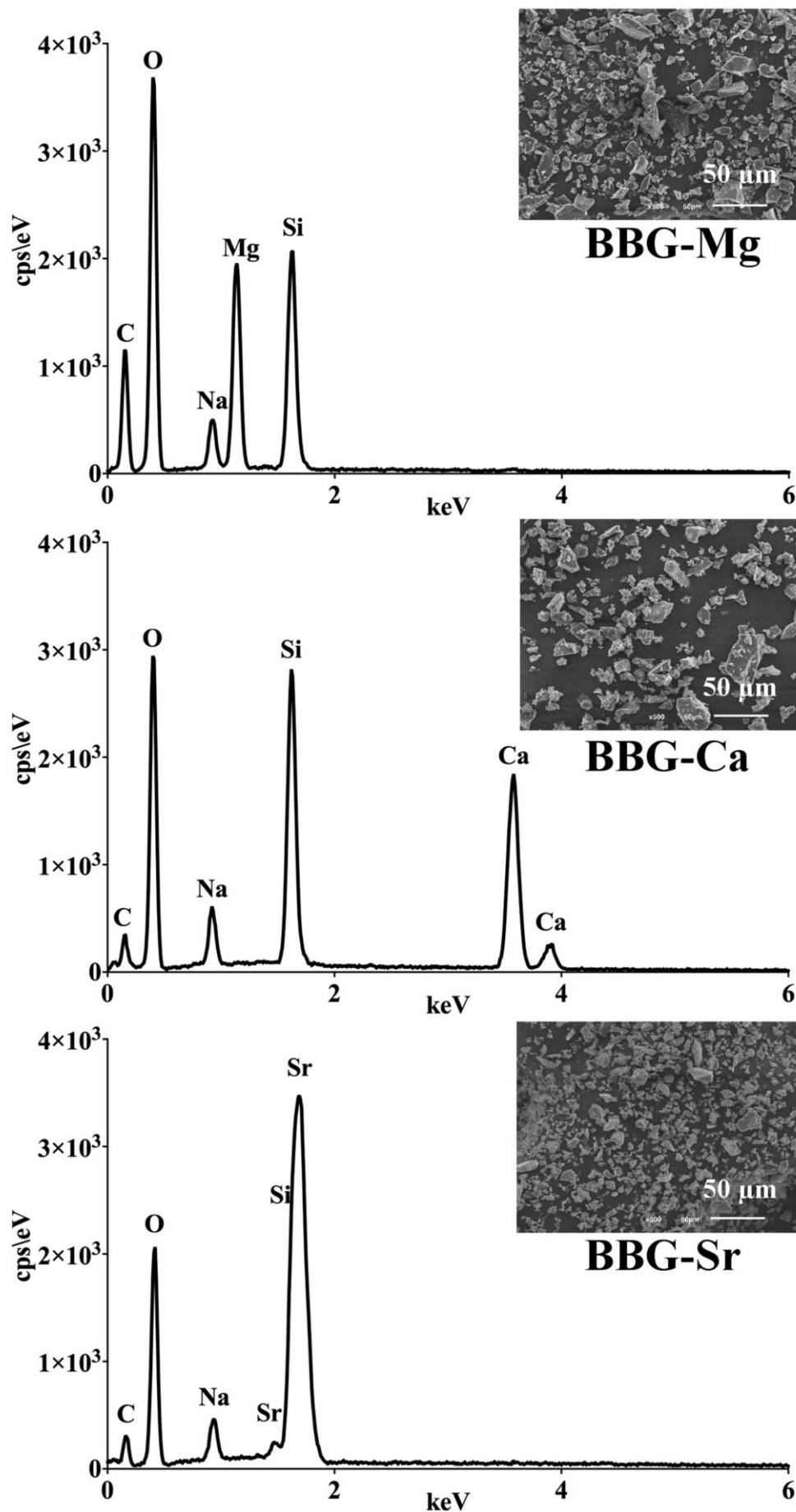
- to commonly used orthopaedic implants. *Journal of Biomedical Materials Research* **62**, 175, 2002.
20. Maeno, S., Niki, Y., Matsumoto, H., Morioka, H., Yatabe, T., Funayama, A., Toyama, Y., Taguchi, T., and Tanaka, J. The effect of calcium ion concentration on osteoblast viability, proliferation and differentiation in monolayer and 3D culture. *Biomaterials* **26**, 4847, 2005.
21. Marie, P.J., Ammann, P., Boivin, G., and Rey, C. Mechanisms of Action and Therapeutic Potential of Strontium in Bone. *Calcif Tissue Int* **69**, 121, 2001.
22. Marie, P.J. Strontium ranelate: A physiological approach for optimizing bone formation and resorption. *Bone* **38**, 10, 2006.
23. Wu, C., Fan, W., Gelinsky, M., Xiao, Y., Simon, P., Schulze, R., Doert, T., Luo, Y., and Cuniberti, G. Bioactive SrO–SiO₂ glass with well-ordered mesopores: Characterization, physiochemistry and biological properties. *Acta Biomaterialia* **7**, 1797, 2011.
24. Gentleman, E., Fredholm, Y.C., Jell, G., Lotfibakhshaiesh, N., O'Donnell, M.D., Hill, R.G., and Stevens, M.M. The effects of strontium-substituted bioactive glasses on osteoblasts and osteoclasts in vitro. *Biomaterials* **31**, 3949, 2010.
25. Santocildes-Romero, M.E., Crawford, A., Hatton, P.V., Goodchild, R.L., Reaney, I.M., and Miller, C.A. The osteogenic response of mesenchymal stromal cells to strontium-substituted bioactive glasses. *Journal of Tissue Engineering and Regenerative Medicine* **9**, 619, 2015.
26. Liang, W., Rahaman, M.N., Day, D.E., Marion, N.W., Riley, G.C., and Mao, J.J. Bioactive borate glass scaffold for bone tissue engineering. *Journal of Non-Crystalline Solids* **354**, 1690, 2008.

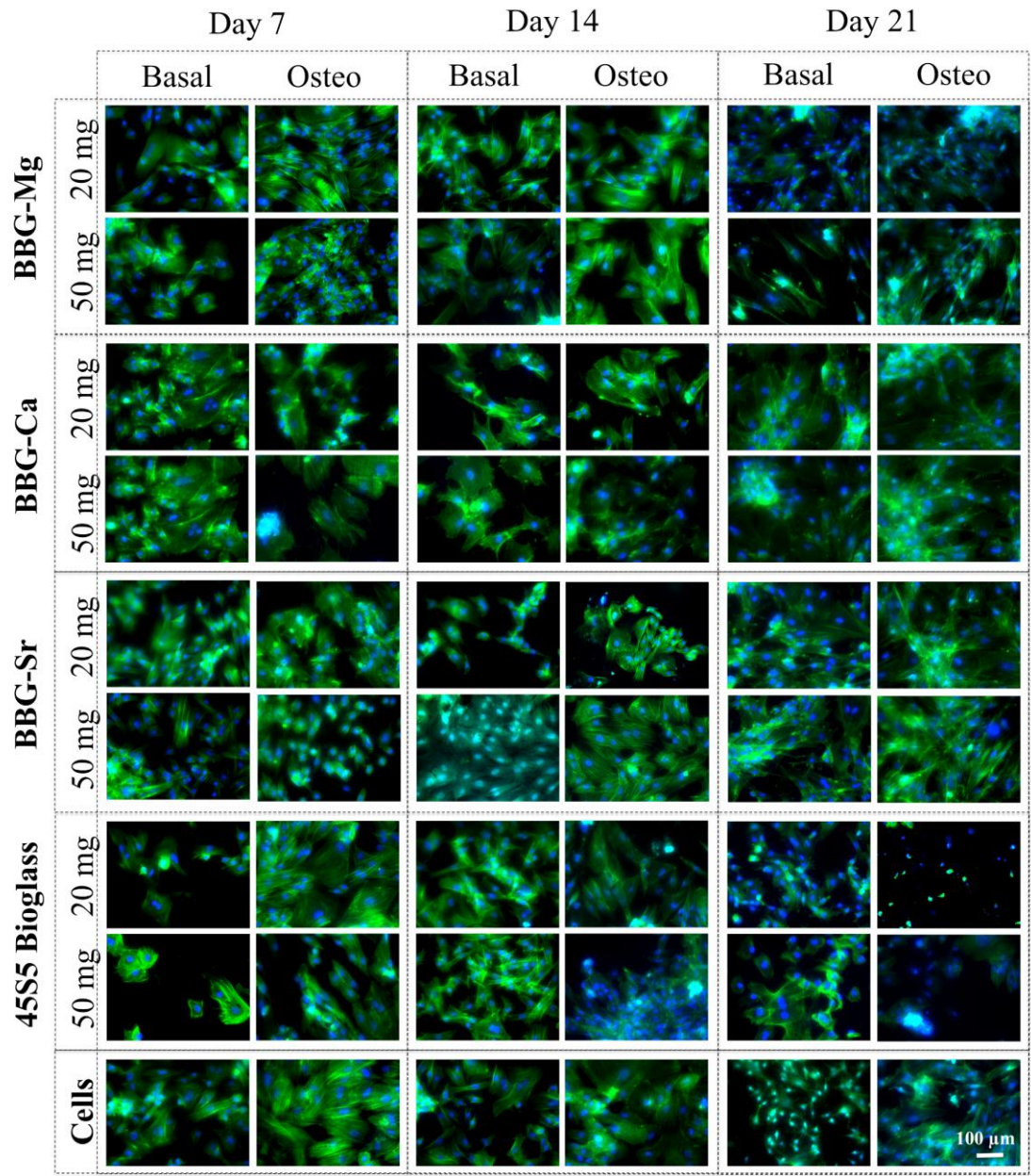
27. Brooke, G., Cook, M., Blair, C., Han, R., Heazlewood, C., Jones, B., Kambouris, M., Kollar, K., McTaggart, S., Pelekanos, R., Rice, A., Rossetti, T., and Atkinson, K. Therapeutic applications of mesenchymal stromal cells. *Seminars in Cell & Developmental Biology* **18**, 846, 2007.
28. Maniopoulos, C., Sodek, J., and Melcher, A.H. Bone formation in vitro by stromal cells obtained from bone marrow of young adult rats. *Cell Tissue Res* **254**, 317, 1988.
29. Fernandes, J.S., Martins, M., Neves, N.M., Fernandes, M.H.F.V., Reis, R.L., and Pires, R.A. Intrinsic Antibacterial Borosilicate Glasses for Bone Tissue Engineering Applications. *ACS Biomaterials Science & Engineering* 2016.
30. Fernandes, J.S., Gentile, P., Moorehead, R., Crawford, A., Miller, C.A., Pires, R.A., Hatton, P.V., and Reis, R.L. Design and Properties of Novel Substituted Borosilicate Bioactive Glasses and Their Glass-Ceramic Derivatives. *Cryst Growth Des* 2016.
31. Stein, G.S., and Lian, J.B. 2 - MOLECULAR MECHANISMS MEDIATING DEVELOPMENTAL AND HORMONE-REGULATED EXPRESSION OF GENES IN OSTEOBLASTS: An Integrated Relationship of Cell Growth and Differentiation. In: Noda M., ed. *Cellular and Molecular Biology of Bone*. San Diego: Academic Press; 1993. pp. 47.
32. Romero, M. The osteogenic response of mesenchymal stromal cells to strontium-substituted bioactive glasses. *Journal of Tissue Engineering and Regenerative Medicine* 2015.
33. Lian, J.B., and Stein, G.S. Development of the osteoblast phenotype: molecular mechanisms mediating osteoblast growth and differentiation. *The Iowa Orthopaedic Journal* **15**, 118, 1995.

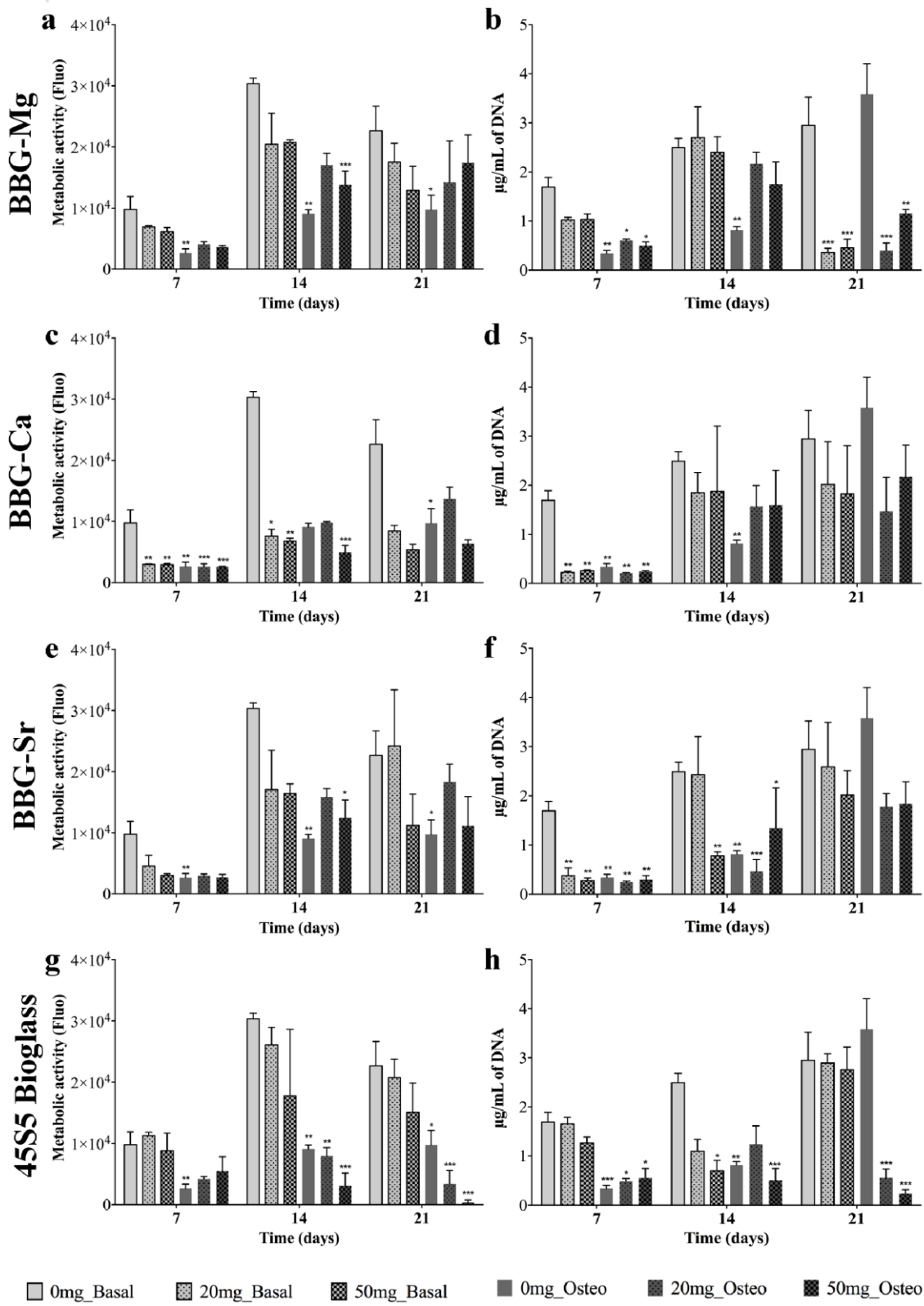
34. Malaval, L., Liu, F., Roche, P., and Aubin, J.E. Kinetics of osteoprogenitor proliferation and osteoblast differentiation in vitro. *Journal of Cellular Biochemistry* **74**, 616, 1999.
35. Huang, S.-C., Wu, B.-C., and Ding, S.-J. Stem cell differentiation-induced calcium silicate cement with bacteriostatic activity. *Journal of Materials Chemistry B* **3**, 570, 2015.
36. Golub, E.E., and Boesze-Battaglia, K. The role of alkaline phosphatase in mineralization. *Current Opinion in Orthopaedics* **18**, 444, 2007.
37. Sabokbar, A., Millett, P.J., Myer, B., and Rushton, N. A rapid, quantitative assay for measuring alkaline phosphatase activity in osteoblastic cells in vitro. *Bone and Mineral* **27**, 57, 1994.
38. Martins, A., Duarte, A.R.C., Faria, S., Marques, A.P., Reis, R.L., and Neves, N.M. Osteogenic induction of hBMSCs by electrospun scaffolds with dexamethasone release functionality. *Biomaterials* **31**, 5875, 2010.
39. Bosetti, M., and Cannas, M. The effect of bioactive glasses on bone marrow stromal cells differentiation. *Biomaterials* **26**, 3873, 2005.
40. Karsenty, G. The genetic transformation of bone biology. *Genes & Development* **13**, 3037, 1999.
41. Amorim, S., Martins, A., Neves, N.M., Reis, R.L., and Pires, R.A. Hyaluronic acid/poly-l-lysine bilayered silica nanoparticles enhance the osteogenic differentiation of human mesenchymal stem cells. *Journal of Materials Chemistry B* **2**, 6939, 2014.
42. Mizuno, M., and Kuboki, Y. Osteoblast-Related Gene Expression of Bone Marrow Cells during the Osteoblastic Differentiation Induced by Type I Collagen. *Journal of Biochemistry* **129**, 133, 2001.

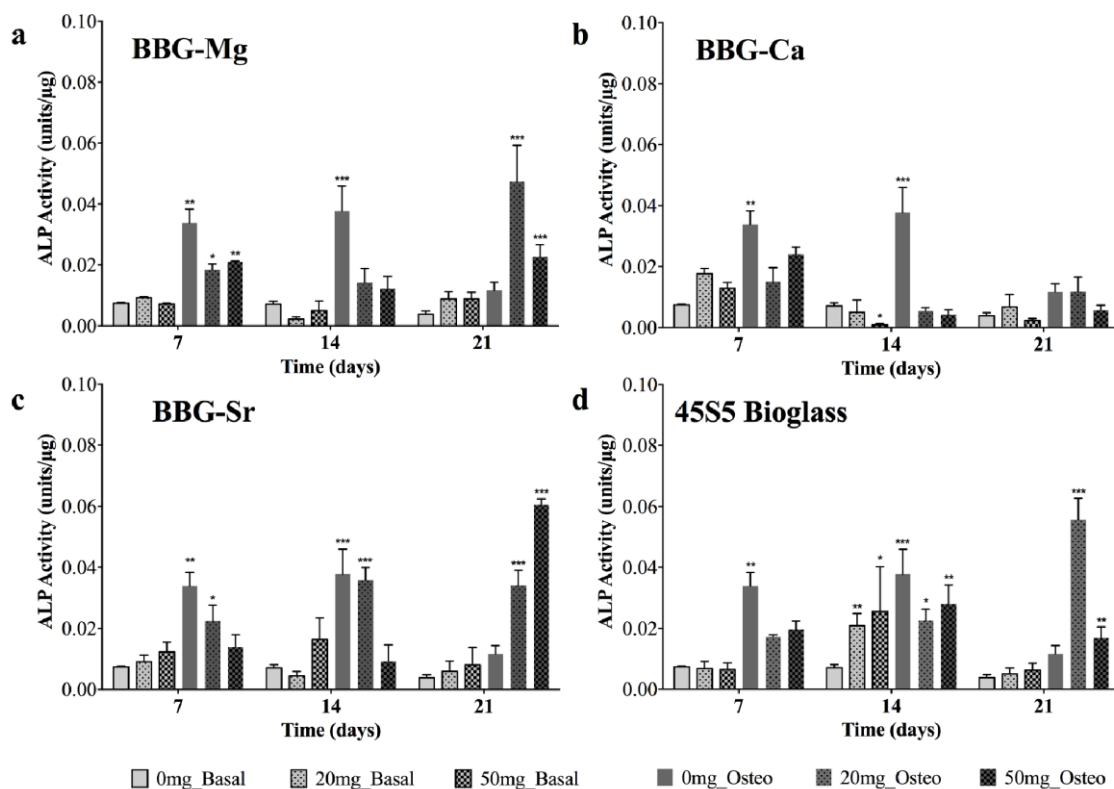
43. Chen, Q.Z., Xu, J.L., Yu, L.G., Fang, X.Y., and Khor, K.A. Spark plasma sintering of sol-gel derived 45S5 Bioglass®-ceramics: Mechanical properties and biocompatibility evaluation. *Materials Science and Engineering: C* **32**, 494, 2012.
44. Chen, X., Liao, X., Huang, Z., You, P., Chen, C., Kang, Y., and Yin, G. Synthesis and characterization of novel multiphase bioactive glass-ceramics in the CaO-MgO-SiO₂ system. *Journal of Biomedical Materials Research Part B: Applied Biomaterials* **93B**, 194, 2010.
45. Hurtel-Lemaire, A.S., Mentaverri, R., Caudrillier, A., Cournarie, F., Wattel, A., Kamel, S., Terwilliger, E.F., Brown, E.M., and Brazier, M. The Calcium-sensing Receptor Is Involved in Strontium Ranelate-induced Osteoclast Apoptosis: NEW INSIGHTS INTO THE ASSOCIATED SIGNALING PATHWAYS. *Journal of Biological Chemistry* **284**, 575, 2009.
46. Habibovic, P., and de Groot, K. Osteoinductive biomaterials—properties and relevance in bone repair. *Journal of Tissue Engineering and Regenerative Medicine* **1**, 25, 2007.

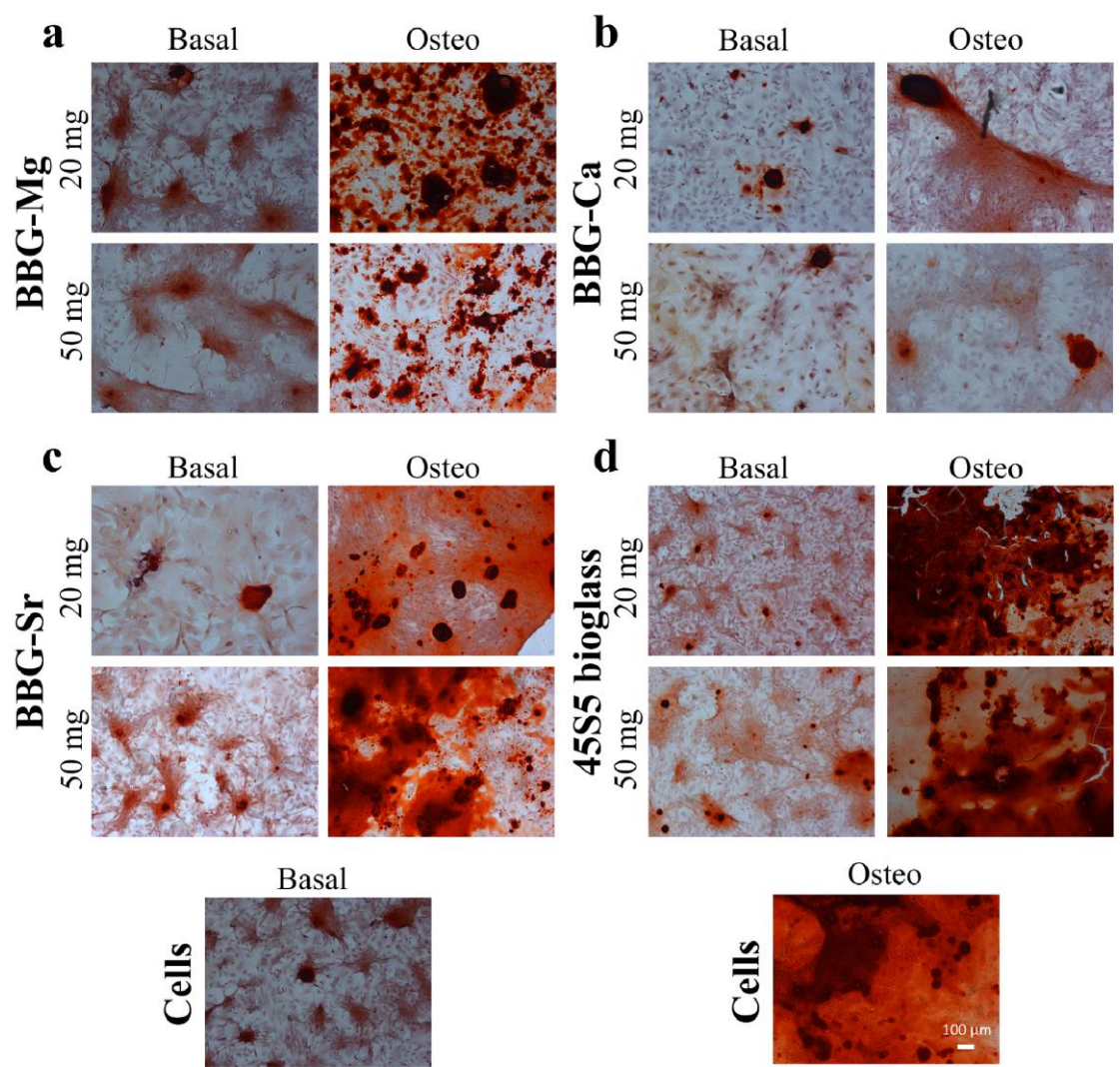
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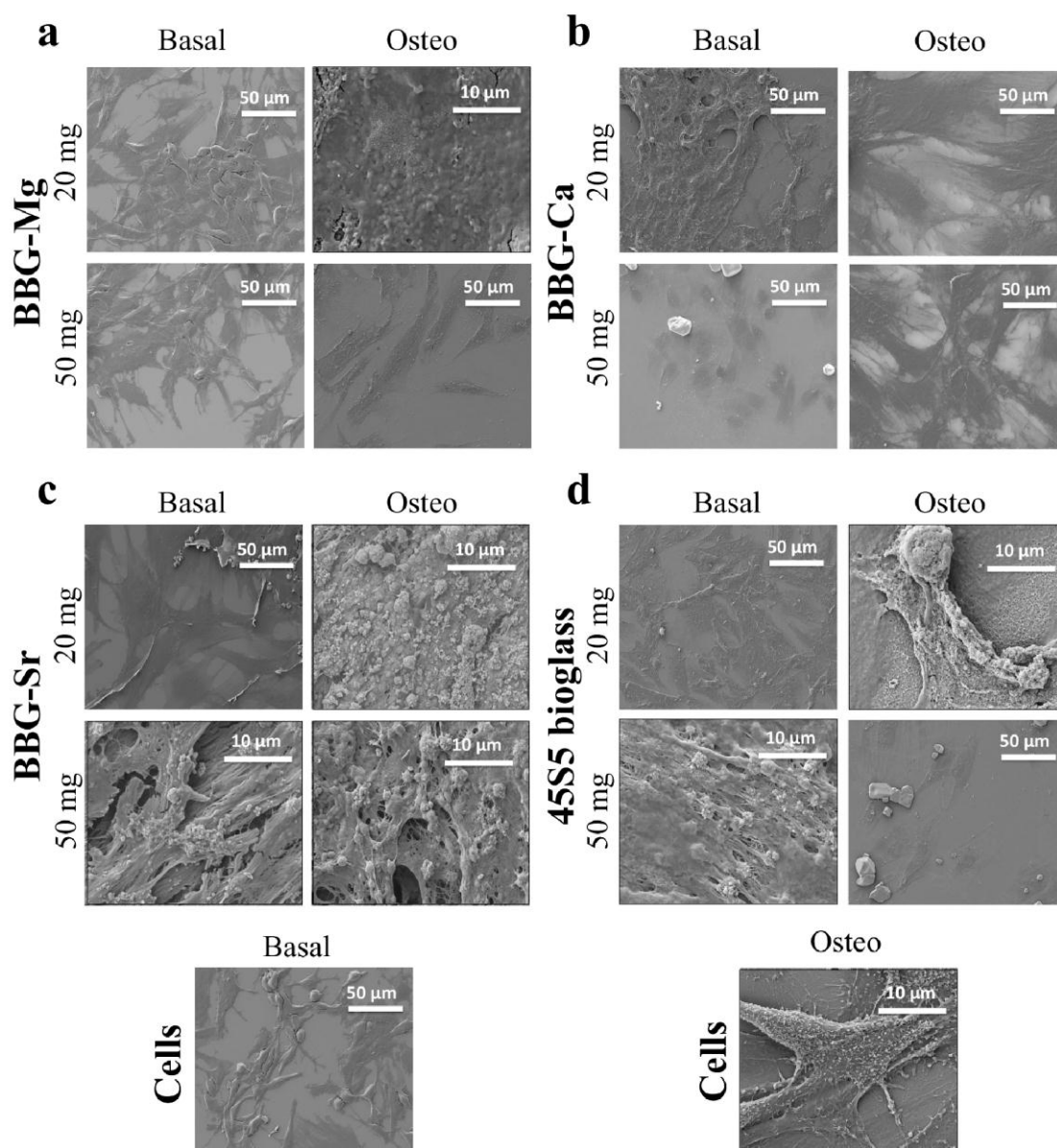


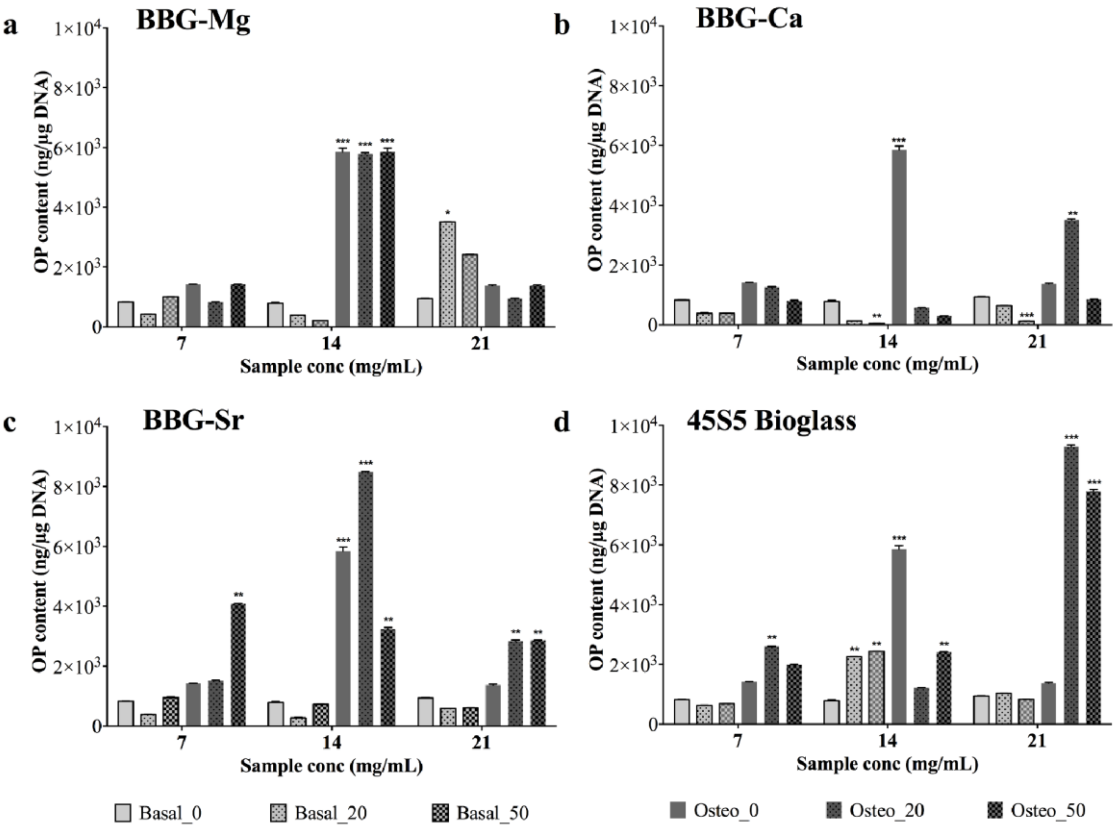












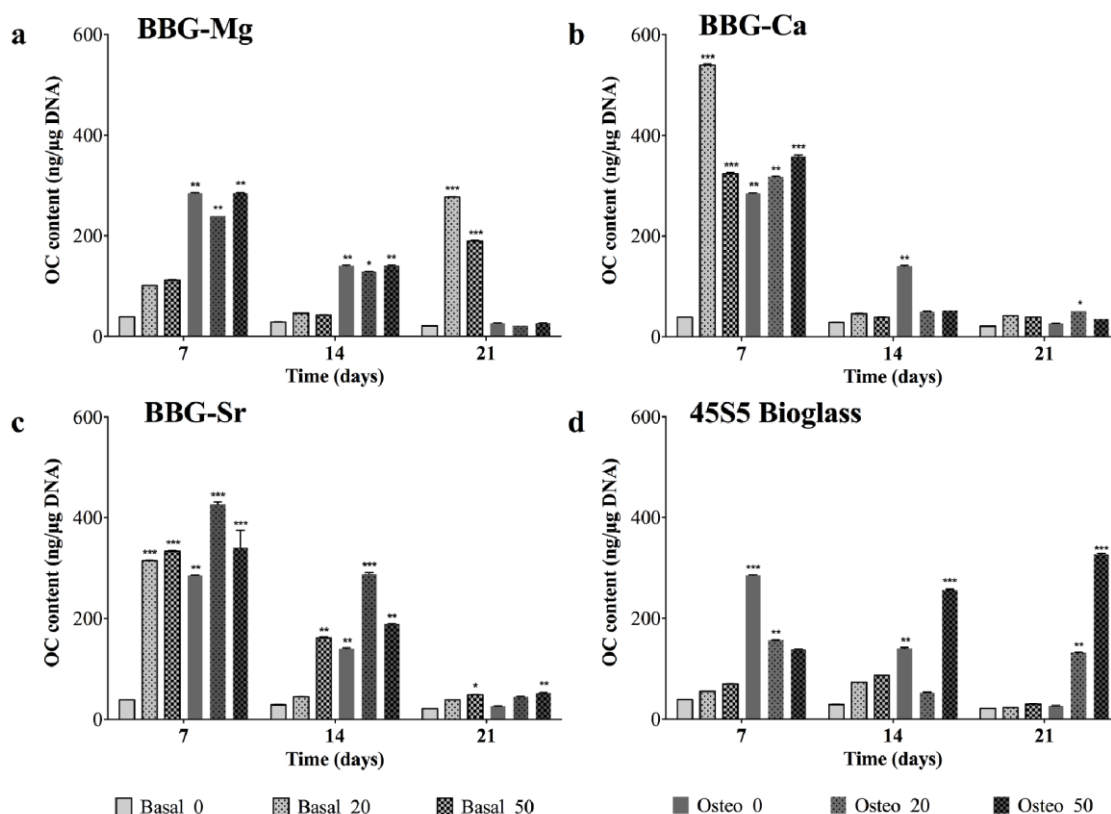


Figure 1. Schematic of the experimental design.

Figure 2. SEM/EDS micrographs of BBGs, a) BBG-Mg, b) BBG-Ca and c) BBG-Sr. SEM images are shown as insets, displaying the morphology of the glass particles. The specific modifier divalent cation is highlighted in yellow for each BBG.

Figure 3. BM-MSCs morphology observed by fluorescence microscopy, after 7, 14 and 21 days culture with BBGs either under basal or osteogenic culture medium. Each sample was incubated at two different concentrations (20 and 50 mg/ml). Cells cultured with basal and osteo medium were used as negative control and 45S5 bioglass[®] incubated with medium was used as positive control. Nuclei stained blue by DAPI; Actin stained green by Phalloidin.

Figure 4. Metabolic activity (PrestoBlue[®] assay) and proliferation (PicoGreen[®] assay) of BM-MSCs cultured either in basal or osteogenic media in the presence of different concentrations (20 and 50 mg/ml) of BBG-Mg (a, b), BBG-Ca (c, d) and BBG-Sr (e, f). The 45S5 bioglass[®] (g, h) was used as control. Standard culture medium was used as negative control. Results are expressed as mean \pm standard deviation with $n = 3$ for each bar. The data was analyzed by non-parametric statistics: Kruskal-Wallis test ($p < 0.0001$), followed by a Dunn's Multiple Comparison test. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ in respect to the control, i.e. BM-MSCs culture in basal media for the respective day of culture (i.e. 7, 14 and 21 days).

Figure 5. ALP activity of BM-MSCs (cultured either in basal or osteogenic media) in the presence of different concentrations (0, 20 and 50 mg/ml) of BBG-Mg (a), BBG-Ca (b) and BBG-Sr (c). The 45S5 bioglass[®] (d) was used as control. Results are expressed as mean \pm standard deviation with $n = 3$ for each bar. The data was analyzed by non-parametric statistics: Kruskal-Wallis test ($p < 0.0001$), followed by a Dunn's Multiple Comparison test. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ in respect to the control, i.e. BM-MSCs culture in basal media for the respective day of culture (i.e. 7, 14 and 21 days).

Figure 6. Alizarin red staining of BM-MSCs cultured during 21 days, either in basal or osteogenic media in the presence of different concentrations (20 and 50 mg/ml) of BBG-Mg (a), BBG-Ca (b) and BBG-Sr (c). The 45S5 bioglass[®] (d) was used as control.

Figure 7. SEM micrographs of BM-MSCs in the presence of different concentrations (20 and 50 mg/ml) of BBG-Mg (a), BBG-Ca (b) and BBG-Sr (c) after 21 days of cell culture either in basal or osteogenic media. Higher SEM magnifications are shown as insets, highlighting the formed apatite-like structures. The 45S5 bioglass[®] was used as a control.

Figure 8. OP protein content of BM-MSCs cultured either with basal or osteogenic media in the presence of different concentrations (20 and 50 mg/ml) of BBG-Mg (a), BBG-Ca (b) and BBG-Sr (c). The 45S5 bioglass® (d) was used as control. Results are expressed as mean \pm standard deviation with $n = 3$ for each bar. The data was analyzed by non-parametric statistics: Kruskal-Wallis test ($p < 0.0001$), followed by a Dunn's Multiple Comparison test. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ in respect to the control, i.e. BM-MSCs culture in basal media for the respective day of culture (i.e. 7, 14 and 21 days).

Figure 9. OC protein content of BM-MSCs cultured either with basal or osteogenic media in the presence of different concentrations (20 and 50 mg/ml) of BBG-Mg (a), BBG-Ca (b) and BBG-Sr (c). The 45S5 bioglass® (d) was used as control. Results are expressed as mean \pm standard deviation with $n = 3$ for each bar. The data was analyzed by non-parametric statistics: Kruskal-Wallis test ($p < 0.0001$), followed by a Dunn's Multiple Comparison test. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ in respect to the control, i.e. BM-MSCs culture in basal media for the respective day of culture (i.e. 7, 14 and 21 days).